	L #	Hits	Search Text	DBs	Time Stamp
1	L1	4018	src	USPAT; US-PGPUB	2002/03/12 10:58
2	L2	529990	activat$8	USPAT; US-PGPUB	2002/03/12 10:59
3	L3	161431	upstream	USPAT; US-PGPUB	2002/03/12 10:59
4	L4	1746	2 near5 3	USPAT; US-PGPUB	2002/03/12 11:18
5	L5	8	4 same 1	USPAT; US-PGPUB	2002/03/12 11:00
6	L6	20	1 same 2 same 3	USPAT; US-PGPUB	2002/03/12 11:01
7	L7	99	1 near2 (activator$1 or activation)	USPAT; US-PGPUB	2002/03/12 11:02
8	L8	1856	hbv or hbx	USPAT; US-PGPUB	2002/03/12 11:02
9	L9	2	7 and 8	USPAT; US-PGPUB	2002/03/12 11:03
10	L10	273	1 near5 2	USPAT; US-PGPUB	2002/03/12 11:19
11	L11	9	8 and 10	USPAT; US-PGPUB	2002/03/12 11:19

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	4018	src	USPAT; US-PGPUB	2002/03/12 10:58
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3	L3	161431	upstream	USPAT; US-PGPUB	2002/03/12 10:59
4	L4	1746	2 near5 3	USPAT; US-PGPUB	2002/03/12 10:59
5	L5	8	4 same 1	USPAT; US-PGPUB	2002/03/12 11:00

US-PAT-NO: 6335010
DOCUMENT-IDENTIFIER: US 6335010 B1
TITLE: Gene therapy in coronary angioplasty and bypass
DATE-ISSUED: January 1, 2002
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Chien; Shu	La Jolla	CA	N/A	N/A
Shyy; John Y-J	San Diego	CA	N/A	N/A

US-CL-CURRENT: 424/93.2,435/320.1 ,435/455 ,514/44
ABSTRACT:

Hemodynamic forces play a key role in inducing 2, theroscler-osis-implicated gene in Vascular endothelial cells. To ellcitate the signal transduction pathway leading to such gene expression, the effects of fluid shearing on the activities of upstream signaling molecules is reported here. Fluid shearing (shear stress=12 dynes/cm.sup.2) induced a transient and rapid activation of p21.sup.ras and preferentially activated c-jun NH.sub.2 terminal kinases (JNK 1, 2) over extracellular signal-regulated kinases (ERK-1, -2). Co-transfection of RasNI7, a dominant negative mutant of Ha-Ras, attenuated the shear-activated JNK and luciferase reporters driven by TPA-responsive elements. JNIK(K-R) and MEKK(K-M), the respective catalytically inactive mutants of JNKI and MEKK, also partially inhibited the shear-induced luciferase reporters. In contrast, Raf301, ERK(K71R), and ERK(K52R), the dominant negative mutants of Raf-1, ERK-1, and ERK-2, respectively, had little effects on the activities of these reporters. The activation of JNK was also correlated with an increased c-Jun transcriptional activity, which was attenuated by a negative mutant of Son of sevenless (Sos). Thus, mechanical stimulation exerted by fluid shearing activates, primarily the Ras-MEKK-JNK pathway in inducing endothelial gene expression.

5 Claims, 25 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 14

DATE FILED: June 30, 1997

---------- KWIC ---------

BSPR:

It is intriguing that Sos can be **upstream to the fluid shearing-activated** p21.sup.ras. The present inventors have found that the negative mutants of GTb2 and Sos can also partially block the shear-induced 4.times.TRE-P1 luc and MCPI-luc-540 in BAEC (23). Grb2 is an adapter protein which contains one **src** homology domain 2 (SH2) and two SH3 domains. GTb2 binds to Sos, a guanine nucleotide exchange factor specific to p21.sup.ras. Thus, the **upstream mechanisms by which the mechanobiochemical transduction activates** Ras pathway may be similar to those for growth factor stimuli. It remains to be investigated how common **upstream signals diverge to activate** JNK (mechanical stimuli) and ERK(growth factor).

US-PAT-NO: 6333341
DOCUMENT-IDENTIFIER: US 6333341 B1
TITLE: Substituted pyridine compounds and methods of use
DATE-ISSUED: December 25, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mantlo; Nathan B.	Lafayette	CO	N/A	N/A
Schlachter; Stephen T.	Boulder	CO	N/A	N/A
Josey; John A.	Longmont	CO	N/A	N/A

US-CL-CURRENT: 514/336,514/256 ,514/318 ,514/886 ,544/242 ,546/193 ,546/268.1
ABSTRACT:

Selected novel substituted pyridine compounds are effective for prophylaxis and treatment of diseases, such as TNF-.alpha., IL-1.beta., IL-6 and/or IL-8 mediated diseases, and other maladies, such as pain and diabetes. The invention encompasses novel compounds, analogs, prodrugs and pharmaceutically acceptable salts thereof, pharmaceutical compositions and methods for prophylaxis and treatment of diseases and other maladies or conditions involving inflammation, pain, diabetes, cancer and the like. The subject invention also relates to processes for making such compounds as well as to intermediates useful in such processes.

11 Claims, 0 Drawing figures
Exemplary Claim Number: 1

DATE FILED: August 21, 2000

---------- KWIC ---------

BSPR:

The present invention also relates to a method of treating cancer which is mediated by Raf and Raf-inducable proteins. Raf proteins are kinases activated in response to extracellular mitogenic stimuli such as PDGF, EGF, acidic FGF, thrombin, insulin or endothelin, and also in response to oncoproteins such as v-**src**, v-sis, and v-fms. Raf functions downstream of ras in signal transduction from the cellular membrane to the nucleus. Compounds in the present invention may be oncolytics through the antagonism of Raf kinase. Antisense constructs which reduce cellular levels of c-Raf and hence Raf activity inhibit the growth of rodent fibroblasts in soft agar, while exhibiting little or no general cytotoxicity. This inhibition of growth in soft agar is highly predictive of tumor responsiveness in whole animals. Moreover Raf antisense constructs have shown efficacy in reducing tumor burden in animals. Examples of cancers where Raf kinase is implicated by overexpression include cancers of the brain, larynx, lung, lymphatic system, urinary tract and stomach, including hystocytic lymphoma, lung adenocarcinoma and small cell lung cancers. Other examples include cancers involving overexpression of **upstream activators** of Raf or Raf-activating oncogenes, including pancreatic and breast carcinoma.

US-PAT-NO: 6255074
DOCUMENT-IDENTIFIER: US 6255074 B1
TITLE: Abl-interactor protein
DATE-ISSUED: July 3, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Pendergast; Ann-Marie	Durham	NC	N/A	N/A
Dai; Zonghan	Englewood	CO	N/A	N/A

US-CL-CURRENT: 435/69.1,435/252.3 ,435/325 ,530/324 ,530/326 ,530/350 ,536/23.5
ABSTRACT:

The present invention relates to a protein that interacts with the cAb1 protein tyrosine kinase and to a nucleic acid sequence encoding same. The invention also relates to complexes of the protein of the invention and cAb1 and to the use of such complexes in the identification of therapeutic and diagnostic agents.

21 Claims, 31 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 18

DATE FILED: July 8, 1998

---------- KWIC ---------

DEPR:
The SH3 domain of the Abi protein of the invention is at the C-terminus (see, for example, aa 346-397 of the Abi protein shown in FIG. 1, A, designated Abi-2). Several proline-rich stretches are present (FIG. 1, B) that constitute binding sites for SH3 domain-containing proteins and contain the consensus PXXP (SEQ ID NO:16) sequence that is present in all high affinity SH3 ligands identified to date (Cohen et al, Cell 80:237 (1995); Rickles et al, EMBO J. 13:5598 (1994)). A polyproline stretch is present **upstream of the SH3 domain which could function as a transcriptional activation** domain (Tanaka et al, Mol. Cell. Biol. 14:6046 (1994)). Sequences enriched in serine/threonine, glutamate/aspartate and proline residues, designated PEST regions, are also found in the present protein. PEST regions are identified in the central and C-terminal portions of the Abi protein (see FIG. 1, B). The N-terminal region of the present protein is basic (eg, calculated pI about 11.4) and homologous to the DNA-binding sequence of homeodomain proteins (the protein of the invention is unique among the family to which it relates in having both an SH3 domain and a homeodomain homologous region). The C-terminal portion of the protein is acidic (eg, pI about 3.5). A serine-rich region is present in the central portion of the protein (see FIG. 1, B). The Abi protein contains several (eg 9) serine/threonine residues followed by proline, indicative of phosphorylation by proline-directed protein kinases (Kemp and Pearson, Trends Biochem. Sci. 15:342 (1990)). Certain sites conform to the cdc 2 kinase consensus sequence Ser/Thr-Pro-X-basic (Moreno and Nurse, Cell 61:549 (1990)). There are also potential cAMP-dependent protein kinase sites (eg 11) and

potential protein kinase C sites (eg 9) (Kemp and Pearson, Trends Biochem. Sci. 15:342 (1990)). Several tyrosines in the sequence are found in peptides that correspond to optimal peptide substrates for the Ab1, Fps and **Src** protein tyrosine kinases (Songyang et al, Nature 373:536 (1995)).

	L #	Hits	Search Text	DBs	Time Stamp
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5	L5	8	4 same 1	USPAT; US-PGPUB	2002/03/12 11:00
6	L6	20	1 same 2 same 3	USPAT; US-PGPUB	2002/03/12 11:01

PGPUB-DOCUMENT-NUMBER: 20020009730
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020009730 A1

TITLE: Human stress array

PUBLICATION-DATE: January 24, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Chenchik, Alex	Palo Alto	CA	US	
Lukashev, Matvey E.	Newton	MA	US	

US-CL-CURRENT: 435/6,536/24.3

ABSTRACT:

Human stress arrays and methods for their use are provided. The subject arrays include a plurality of polynucleotide spots, each of which is made up of a polynucleotide probe composition of unique polynucleotides corresponding to a human stress gene. The subject arrays find use in hybridization assays, particularly in assays for the identification of differential gene expression of human stress genes.

DATE FILED: February 13, 2001

---------- KWIC ---------

DETL:
ST106 protein component 1 (TEP1) U86136 Q99973 pentraxin 3 (PTX3); pentaxin-related gene rapidly ST108 induced by IL1-beta X63613 P26022 neuronal DNAJ-like heat shock protein 1 (HSJ1); ST109 40-kDa heat shock protein 3 (HSPF3) X63368 P25686 dual specificity phosphatase 1 (DUSP1); CL100; PTPN10; HVHI; MAP kinase phosphatase 1 ST110 (MKP1) U01669; X68277 P28562 Q15853; Q15852; fos-interacting **upstream** stimulatory factor 2 Q00671; Q00709; ST111 (USF2); FIP Y07661 Q05750; Q07952 insulin promoter factor 1 (IPF1); islet/duodenum homeobox protein 1 (IDX1); somatostatin transcription factor 1 (STF1); maturity-onset ST112 diabetes of the young protein IV (MODY4); X99894 P52945; Q60594 pancreas/duodenum homeobox protein 1 (PDX1) ST113 inhibitor of DNA-binding protein 4 (ID4) U28368 P47928; Q13005 51114 thrombospondin 4 (THBS4) Z19585 P35443 ST115 cyclin F (CCNF) Z36714 P41002 63-kDa FK506-binding protein 9 (FKBP9); FKBP63; ST117 FKBP60 AF089745 Q95302 ST118 FK506-binding protein AF092137 Q9Y6B0 peroxisomal biogenesis factor 11 alpha (PEX11- ST119 alpha; PEXilA) AF093668 Q75192 cysteine desulfurase; nitrogen-fixing bacteria S ST120 homolog (N1FS) AF097025 Q9Y697 cathelicidin antimicrobial peptide (CAMP); FALL39; ST121 CAP18 Z38026 P49913 ST122

caspase 14 (CASP14); MICE AF097874 Q95823 ST123 timeless homolog (TIM) AF098162 Q9UNS1 ST124 period homolog 2 (PER2) AB002345 Q15055 ST125 BTB & CNC homology protein 1 (BACH1) AB002803 Q14867; Q43285 sex-determining region Y box-containing gene 20 ST126 (SRY box-containing gene 20; SOX20) AB006867 Q60248 heat shock transcription factor 2-binding protein ST127 (HSF2BP) AB007131 Q75031 transforming growth factor beta 1-induced transcript ST128 1 (TGFBII1); HICS; ARA55; TSC5 AB007836 Q43294 ST130 KIAA0516 AB011088 Q60271 DEAD/H (Asp-Glu-Ala-Aspf His) box polypeptide 16 ST131 (DDX16) AB011149 Q60231; Q60322 O75771; O60355; O43537; O76085; ST132 RAD51-like protein 3 (RAD5IL3); TRAD AF034956 Q75196; Q75847 ST133 aquaporin 8 (AQP8) AB013456 Q94778 ST134 KIAA0700 AB014600 O75182 KIAA0709; macrophage mannose endocytic ST135 receptor AB014609 Q9UBG0 ST136 MRJ member of the DNAJ protein family AB014888 Q75190; Q95806 adaptor-related protein complex 1 sigma 2 subunit (AP1S2); clathrin adaptor complex 1 sigma IB ST137 subunit AB015320 none ST139 KIAAI027; talin (TLN) AB028950 Q9UPX3 phosphatidylethanolamine N-methyltransferase ST140 (PEMT) AB029821 Q9Y6V9 ST141 choline kinase-like protein (CHKL) AB029886 Q9Y259; Q13388 degenerative spermatocyte homolog (DEGS); ST142 membrane fatty acid (lipid) desaturase (MLD) AF002668 Q15121 estrogen receptor-binding fragment-associated gene 9 (EBAG9; EB9); receptor-binding cancer ST143 antigen expressed on 5150 cells 1 (RCAS1) AF006265 Q00559 ST144 outer dense fiber of sperm tails protein 2 (ODF2) AF012549 Q14721 ST146 palmitoyl-protein thioesterase 2 (PPT2) AF020544 Q14799 eukaryotic translation initiation factor 3 subunit 4 ST147 (EIF3S4); EIF3-delta AF020833 Q75821; Q14801 ST148 DJ-1 RNA-binding protein regulatory subunit AF021819 Q14805 ST149 period homolog 1 (PER1); RIGU1 AF022991 Q15534 ST150 deleted in liver cancer protein 1 (DLC1) AF026219 Q14868 branched chain alpha-ketoacid dehydrogenase ST151 kinase (BOKOK) AF026548 Q14874 ST152 microsomal glutathione 5-transferase 3 (MGST3) AF026977 Q14880 ST153 glypican 4 (GPC4) AF030186 O75487; Q9UPD9 testis-enhanced gene transcript (TEGT); BAX ST154 inhibitor 1 (BI1) AF033095 P55061; Q14938 ST155 stromal cell-derived factor receptor 1 (SDFR1) AF035287 Q9Y499 ST156 RING zinc finger protein 13 (RNF13; RZF) AF037204 Q43567 ST157 voltage-dependent anion channel 3 (VDAC3) AF038962 Q9Y277; Q9UISO ST158 cell death-inducing OFFA-like effector A (CIDEA) AF041378 O60543 ST1 59 unc51 -like kinase 1 homolog (ULK1) AF045458 Q75385 ubiquitin-**activating** enzyme EIC (UBEIC); UBA3 ST160 homolog AF046024 Q76088 amyloid beta A4 protein-binding family A member 2 (APBA2); X11-like protein (XIL); Munc18-1- ST161 interacting protein 2 (MINT2) AF047348 Q99767; Q60571 ST162 32-kDa thioredoxin-like protein (TXNL; TXL) AF051896 Q43396 ST163 Sec6l-gamma (SEC61G) AF054184 none microsomal NAD+-dependent retinol ST164 dehydrogenase 4 (RODH4) AF057034 Q75452 serine protease 21 (PRSS2 1); testisin 1 (TEST1); ST165 serine protease from eosinophils 1 (ESP1) AF058300 Q9Y6M0 mitochondrial ribosomal protein SI 2 (RPMS12; ST166 RPSM12) AF058761 Q15235 ADP-forming succinate-CoA ligase beta subunit ST167 (SUCLA2) AF058953 Q95194 ST168 E2F transcription factor 6 (E2F6) AF059292 Q75461; Q60544 ST169 UDP-glucose dehydrogenase (UDPGDH; UGDH) AF061016 Q60701; Q60589 catenin delta 1 (CTNND 1); cadherin-associated **src** ST170 substrate (GAS) AF062333 Q60935 heart & neural crest derivatives-expressed protein 1 ST171 (HAND1); THING1 AF061756 Q96004 clone 24651 (IMAGE Consortium human infant ST172 brain library IN1B) AF070648 none ST173 SURF-4 AF078866 Q9UNZ1 ST174 growth factor-independent protein 1B (GFI1B) AF081946 Q95270 D-type cyclin-interacting protein 1 (DIP1); GCIP; ST175 MAID AF082569 Q95273 thyroid receptor-1nteracting protein 15 (TRIP 15); ST176 ALIEN; SGN2

AF084260 none Housekeeping Genes liver glyceraldehyde 3-phosphate dehydrogenase 16 (GAPDH; G3PDH) X01677 P04406 P02570; Q11211; P99021; Q64316; 49 cytoplasmic beta-actin (ACTB) X00351 P70514 901 tubuiin alpha 1 (TUBA1) K00558 P04687 902 major histocompatibility complex class 1 C (HLAC) M11886 P10321 23-kDa highly basic protein; 60S ribosomal protein 903 L13A(RPL13A) X56932 P40429 904 40S ribosomal protein 39 (RPS9) U14971 P46781 905 ubiquitin C (UBC) M26880 none phospholipase A2; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase **activation** protein zeta polypeptide (YWHAZ); 14-3- 3 protein zeta/delta; protein kinase C inhibitor protein 1 (K01P1); factor **activating** exoenzyme S 906 (FAS) M86400 P29312; P29213 hypoxanthine-guanine phosphoribosyltransferase 1 907 (HPRT1) V00530 P00492

PGPUB-DOCUMENT-NUMBER: 20010014734
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20010014734 A1

TITLE: PROGRESSION ELEVATED GENE-3 AND USES THEREOF

PUBLICATION-DATE: August 16, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
FISHER, PAUL B.	SCARSDALE	NY	US	

US-CL-CURRENT: 536/23.1,424/93.1 ,435/320.1

ABSTRACT:

This invention provides a vector suitable for introduction into a cell, comprising: a) an inducible PEG-3 regulatory region; and b) a gene encoding a product that causes or may be induced to cause the death or inhibition of cancer cell growth. In addition, this invention further provides the above-described vectors, wherein the inducible PEG-3 regulatory region is a promoter. This invention further provides the above-described vectors, wherein the gene encodes an inducer of apoptosis. In addition, this invention provides the above-described vectors, wherein the gene is a tumor suppressor gene. In addition, this invention provides the above-described vectors, wherein the gene encodes a viral replication protein. This invention also provides the above-described vectors, wherein the gene encodes a product toxic to cells or an intermediate to a product toxic to cells. In addition, this invention provides the above-described vectors, wherein the gene encodes a product causing enhanced immune recognition of the cell. This invention further provides the above-described vectors, wherein the gene encodes a product causing the cell to express a specific antigen.

DATE FILED: March 31, 1998

---------- KWIC ---------

DETX:
[0557] Nuclear run-on assays indicate that PEG-3 expression directly correlates with an increase in the rate of RNA transcription (17). This association is supported by the isolation of a genomic fragment **upstream** of the 5' untranslated region of the PEG-3 cDNA and demonstration that this sequence linked to a luciferase reporter gene is **activated** as a function of cancer progression, oncogenic transformation and DNA damage (FIGS. 15, 16 & 17). Additionally, changes in the stability of PEG-3 mRNA may also contribute to differential expression of this gene as a function of cancer progression,

oncogene expression and DNA damage. To address this issue mRNA stability (RNA degradation) assays will be performed as described in detail previously (43). Our analysis focuses on the effect of cancer progression (E11-NMT, R1 and R2 cells), oncogenic transformation (Ha-ras, V-**src**, H5hr1 and HPV-18 transformed CREF cells) and DNA damage (gamma irradiation and MMS-treatment of CREF cells). Appropriate controls, E11, untransformed CREF cells and CREF cells not treated with DNA damaging agents, respectively, and experimental samples will be incubated without additions or in the presence of 5 mg/ml of actinomycin D (in the dark), and 30, 60 and 120 min later, total cellular RNA will be isolated and analyzed for gene expression using Northern hybridization. RNA blots will be quantitated by densitometric analysis using a Molecular Dynamics densitometer (Sunnyvale, Calif.). These straight forward experiments will indicate if the stability of PEG-3 is altered in cells as a direct consequence of spontaneous progression, expression of defined oncogenes or as a consequence of DNA damage.

DETX:
[0683] Nuclear run-on assays indicate that PEG-3 expression directly correlates with an increase in the rate of RNA transcription (17). This association is supported by the isolation of a genomic fragment **upstream** of the 5' untranslated region of the PEG-3 cDNA and demonstration that this sequence linked to a luciferase reporter gene is **activated** as a function of cancer progression, oncogenic transformation and DNA damage. Additionally, changes in the stability of PEG-3 mRNA may also contribute to differential expression of this gene as a function of cancer progression, oncogene expression and DNA damage. To address this issue mRNA stability (RNA degradation) assays will be performed as described in detail previously (32). Our analysis will focus on the effect of cancer progression (E11-NMT, R1 and R2 cells), oncogenic transformation (Ha-ras, V-**src**, H5hr1 and HPV-18 transformed CREF cells) and DNA damage (gamma irradiation and MMS-treatment of CREF cells). Appropriate controls, E11, untransformed CREF cells and CREF cells not treated with DNA damaging agents, respectively, and experimental samples will be incubated without additions or in the presence of 5 .mu.g/ml of actinomycin D (in the dark), and 30, 60 and 120 min later, total cellular RNA will be isolated and analyzed for gene expression using Northern hybridization. RNA blots will be quantitated by densitometric analysis using a Molecular Dynamics densitometer (Sunnyvale, Calif.) (32). These straight forward experiments will indicate if the stability of PEG-3 is altered in cells as a direct consequence of spontaneous progression, expression of defined oncogenes or as a consequence of DNA damage.

US-PAT-NO: 6335010
DOCUMENT-IDENTIFIER: US 6335010 B1
TITLE: Gene therapy in coronary angioplasty and bypass
DATE-ISSUED: January 1, 2002
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Chien; Shu	La Jolla	CA	N/A	N/A
Shyy; John Y-J	San Diego	CA	N/A	N/A

US-CL-CURRENT: 424/93.2,435/320.1 ,435/455 ,514/44
ABSTRACT:

Hemodynamic forces play a key role in inducing 2, theroscler-osis-implicated gene in Vascular endothelial cells. To ellcitate the signal transduction pathway leading to such gene expression, the effects of fluid shearing on the activities of upstream signaling molecules is reported here. Fluid shearing (shear stress=12 dynes/cm.sup.2) induced a transient and rapid activation of p21.sup.ras and preferentially activated c-jun NH.sub.2 terminal kinases (JNK 1, 2) over extracellular signal-regulated kinases (ERK-1, -2). Co-transfection of RasNI7, a dominant negative mutant of Ha-Ras, attenuated the shear-activated JNK and luciferase reporters driven by TPA-responsive elements. JNIK(K-R) and MEKK(K-M), the respective catalytically inactive mutants of JNKI and MEKK, also partially inhibited the shear-induced luciferase reporters. In contrast, Raf301, ERK(K71R), and ERK(K52R), the dominant negative mutants of Raf-1, ERK-1, and ERK-2, respectively, had little effects on the activities of these reporters. The activation of JNK was also correlated with an increased c-Jun transcriptional activity, which was attenuated by a negative mutant of Son of sevenless (Sos). Thus, mechanical stimulation exerted by fluid shearing activates, primarily the Ras-MEKK-JNK pathway in inducing endothelial gene expression.

5 Claims, 25 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 14

DATE FILED: June 30, 1997

---------- KWIC ---------

BSPR:

It is intriguing that Sos can be **upstream** to the fluid shearing-**activated** p21.sup.ras. The present inventors have found that the negative mutants of GTb2 and Sos can also partially block the shear-induced 4.times.TRE-P1 luc and MCPI-luc-540 in BAEC (23). Grb2 is an adapter protein which contains one **src** homology domain 2 (SH2) and two SH3 domains. GTb2 binds to Sos, a guanine nucleotide exchange factor specific to p21.sup.ras. Thus, the **upstream** mechanisms by which the mechanobiochemical transduction **activates** Ras pathway may be similar to those for growth factor stimuli. It remains to be investigated how common **upstream** signals diverge to **activate** JNK (mechanical stimuli) and ERK(growth factor).

US-PAT-NO: 6333341
DOCUMENT-IDENTIFIER: US 6333341 B1
TITLE: Substituted pyridine compounds and methods of use
DATE-ISSUED: December 25, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mantlo; Nathan B.	Lafayette	CO	N/A	N/A
Schlachter; Stephen T.	Boulder	CO	N/A	N/A
Josey; John A.	Longmont	CO	N/A	N/A

US-CL-CURRENT: 514/336,514/256 ,514/318 ,514/886 ,544/242 ,546/193 ,546/268.1
ABSTRACT:

Selected novel substituted pyridine compounds are effective for prophylaxis and treatment of diseases, such as TNF-.alpha., IL-1.beta., IL-6 and/or IL-8 mediated diseases, and other maladies, such as pain and diabetes. The invention encompasses novel compounds, analogs, prodrugs and pharmaceutically acceptable salts thereof, pharmaceutical compositions and methods for prophylaxis and treatment of diseases and other maladies or conditions involving inflammation, pain, diabetes, cancer and the like. The subject invention also relates to processes for making such compounds as well as to intermediates useful in such processes.

11 Claims, 0 Drawing figures
Exemplary Claim Number: 1

DATE FILED: August 21, 2000

---------- KWIC ---------

BSPR:

The present invention also relates to a method of treating cancer which is mediated by Raf and Raf-inducable proteins. Raf proteins are kinases **activated** in response to extracellular mitogenic stimuli such as PDGF, EGF, acidic FGF, thrombin, insulin or endothelin, and also in response to oncoproteins such as v-**src**, v-sis, and v-fms. Raf functions downstream of ras in signal transduction from the cellular membrane to the nucleus. Compounds in the present invention may be oncolytics through the antagonism of Raf kinase. Antisense constructs which reduce cellular levels of c-Raf and hence Raf activity inhibit the growth of rodent fibroblasts in soft agar, while exhibiting little or no general cytotoxicity. This inhibition of growth in soft agar is highly predictive of tumor responsiveness in whole animals. Moreover Raf antisense constructs have shown efficacy in reducing tumor burden in animals. Examples of cancers where Raf kinase is implicated by overexpression include cancers of the brain, larynx, lung, lymphatic system, urinary tract and stomach, including hystocytic lymphoma, lung adenocarcinoma and small cell lung cancers. Other examples include cancers involving overexpression of **upstream activators** of Raf or Raf-**activating** oncogenes, including pancreatic and breast carcinoma.

US-PAT-NO: 6300111
DOCUMENT-IDENTIFIER: US 6300111 B1
TITLE: Constitutively active phosphatidylinositol 3-kinase and uses thereof
DATE-ISSUED: October 9, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Klippel; Anke	San Francisco	CA	N/A	N/A
Williams; Lewis T.	Tiburon	CA	N/A	N/A

US-CL-CURRENT: 435/194,435/252.3 ,435/320.1 ,435/325 ,536/23.2
ABSTRACT:

The invention provides a method of producing a constitutively active phosphatidylinositol 3-kinase (PI 3-kinase) comprising the catalytic p110 subunit covalently attached at the N-terminus to the iSH2 region of the regulatory subunit, p85. The invention discloses one form of the constitutively active kinase, p110*, which functions independently of growth factor stimulation. Expression vectors encoding a constitutively active PI 3-kinase and cells containing such expression vectors are provided. The invention also provides methods of using the constitutively active phosphatidylinositol 3-kinase to generate phosphoinositides, to identify cellular target proteins and associating molecules of PI 3-kinase, to screen for inhibitors of PI 3-kinase activity and to treat certain diseases, in particular, proliferative diseases. Kits comprising the constitutively active kinase are also provided.
8 Claims, 10 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

DATE FILED: March 10, 1999

---------- KWIC ---------

BSPR:
Phosphatidylinositol 3-kinase is one of many activities stimulated by growth factors. Phosphatidylinositol 3-kinase is known to be involved in the regulation of cell growth and oncogenic transformation (Cantley et al., Cell, 64:1657 (1993)). The enzyme is found associated with receptor protein tyrosine kinases such as PDGF-R-.beta., CSF-1 receptor, Insulin receptor and IGF-1 receptor as well as non-receptor tyrosine kinase oncogenes, e.g., **src,** gag-abl and fyn. Studies on mutants of platelet-derived growth factor (PDGF) receptor have shown that phosphatidylinositol 3-kinase is a key mediator of PDGF-mediated mitogenic signaling (Fantl et al., Cell, 69:413 (1992); Valius et al., ibid., 73:321 (1993)). PDGF-R mutants that are unable to bind phosphatidylinositol 3-kinase are also unable to induce a mitogenic response after growth factor stimulation and unable to **activate** p21.sup.c-ras (ras). These data suggested that phosphatidylinositol 3-kinase acts **upstream** of ras in PDGF-stimulated signaling.

US-PAT-NO: 6296848
DOCUMENT-IDENTIFIER: US 6296848 B1
TITLE: GRB2 associating polypeptides and nucleic acids encoding therefor
DATE-ISSUED: October 2, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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US-CL-CURRENT: 424/94.5,435/194 ,435/21 ,435/252.3 ,435/320.1 ,435/69.1 ,530/300 ,530/350 ,536/23.2
ABSTRACT:
The present invention generally relates to novel GRB2 associating proteins and nucleic acids which encode these protein. In particular, these novel proteins possess inositol polyphosphate 5-phosphatase and phosphatidylinositol 5-phosphatase activities, important in growth factor mediated signal transduction. As such, the proteins, nucleic acids encoding the proteins, cells capable of expressing these nucleic acids and antibodies specific for these proteins will find a variety of uses in a variety of screening, therapeutic and other applications.
14 Claims, 31 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 27

DATE FILED: October 14, 1999

---------- KWIC ---------

DEPR:
PtdIns(3,4,5)P.sub.3 in particular, is the product of phosphatidyl inositol 3-kinase ("PI3 kinase"), an important agonist **activated** signaling protein, stimulated in growth factor mediated signal transduction. PI3-kinase is known to be involved in the regulation of cell growth and oncogenic transformation (Cantley et al., Cell, 64:1657 (1993)). Upon growth factor receptor stimulation, the wild-type PI3-kinase is **activated** and can phosphorylate phosphatidylinositol ("PtdIns") at the 3' position of the inositol ring. These phosphatidylinositol 3-phosphates are candidate second messenger molecules. The PI3-kinase enzyme is found associated with receptor protein tyrosine kinases such as PDGF-R-.beta., CSF-1 receptor, Insulin receptor and IGF-1 receptor as well as non-receptor tyrosine kinase oncogenes, e.g., **src,** gag-abl and fyn. Studies on mutants of platelet-derived growth factor (PDGF) receptor have shown that PI3-kinase is a key mediator of PDGF-mediated mitogenic signaling (Fantl et al., Cell, 69:413 (1992); Valius et al., ibid., 73:321 (1993)). PDGF-R mutants that are unable to bind PI3-kinase are also unable to induce a mitogenic response after growth factor stimulation and unable to **activate** p2lc-Ras (Ras). These data indicate that PI3-kinase acts **upstream** of

Ras in PDGF-stimulated signaling. Studies also indicate that the PI3-kinase product, PtdIns(3,4,5)P.sub.3 is not the final product produced during the initial phases of signaling, indicating further processing of this signaling molecule. Stephens, et al., Nature 351:33-39 (1991), Hawkins, et al., Nature 358:157-159 (1992).

US-PAT-NO: 6268173
DOCUMENT-IDENTIFIER: US 6268173 B1
TITLE: Polynucleotide encoding transcriptional intermediary factor-2
DATE-ISSUED: July 31, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Chambon; Pierre	Blaesheim	N/A	N/A	FRX
Gronemeyer; Hinrich	Oberkirch	N/A	N/A	DEX
Voegel; Johannes	Strasbourg	N/A	N/A	FRX

US-CL-CURRENT: 435/69.1,435/320.1 ,435/325 ,536/23.1 ,536/23.5
ABSTRACT:

The present invention concerns a nuclear receptor (NR) transcriptional mediator. More specifically, isolated nucleic acid molecules are provided encoding transcriptional intermediary factor-2 (TIF2). Recombinant methods for making TIF2 polypeptides are also provided as are TIF2 antibodies. Screening methods are also provided for identifying agonists and antagonists of the activation function AF-2 of nuclear receptors, for identifying agonists and antagonists of the AD1 activation domain activity of TIF2, and for identifying agonists and antagonists of the AD2 activation domain activity of TIF2.

38 Claims, 63 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 46

DATE FILED: July 11, 1997

---------- KWIC ---------

DEPR:
While the internal deletion of residues Asp.sub.1061 to Ala.sub.1070 (TIF2.19) had only a minor effect on the ability of TIF2.13 to transactivate, deletion of the Glu.sub.1071 to Leu.sub.1080 segment (mutant TIF2.20) significantly reduced TIF2 AD1 transcriptional activity. Notably, these residues belong to a sequence predicted to fold into an amphipathic .alpha.-helical structure which is highly conserved between TIF2 and **SRC**-1 (FIG. 9a). The involvement of this region in transactivation was confirmed by analysis of mutants TIF2.21 to TIF2.32 (FIGS. 9a and b). All constructs containing the TIF2 wild-type sequence from Asp.sub.1075 to Leu.sub.1087 stimulated transcription, whereas even a deletion of only some of these residues significantly reduced transcriptional **activation**. However, on its own this .alpha.-helical peptide transactivated very poorly, and had to be incorporated into additional **upstream** and/or downstream TIF2 sequences to generate significant transcriptional activity (FIGS. 9a and b; compare mutants TIF2.13, TIF2.21 and TIF2.32). Importantly, in all cases ADD activity coincided with CBP interaction, since transcriptionally inactive constructs did not interact with CBP (TIF2.24, TIF2.27 and TIF2.29 in FIGS. 9a-c), while transcriptionally active mutants also bound CBP. Moreover, the strength of the in vitro interaction with GST-CBP apparently correlated with transactivation efficiency (FIGS. 9a-c; e.g.,

compare TIF2.21 and TIF2.3 1).

US-PAT-NO: 6255074
DOCUMENT-IDENTIFIER: US 6255074 B1
TITLE: Abl-interactor protein
DATE-ISSUED: July 3, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Pendergast; Ann-Marie	Durham	NC	N/A	N/A
Dai; Zonghan	Englewood	CO	N/A	N/A

US-CL-CURRENT: 435/69.1,435/252.3 ,435/325 ,530/324 ,530/326 ,530/350 ,536/23.5
ABSTRACT:

The present invention relates to a protein that interacts with the cAb1 protein tyrosine kinase and to a nucleic acid sequence encoding same. The invention also relates to complexes of the protein of the invention and cAb1 and to the use of such complexes in the identification of therapeutic and diagnostic agents.

21 Claims, 31 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 18

DATE FILED: July 8, 1998

---------- KWIC ---------

DEPR:
The SH3 domain of the Abi protein of the invention is at the C-terminus (see, for example, aa 346-397 of the Abi protein shown in FIG. 1, A, designated Abi-2). Several proline-rich stretches are present (FIG. 1, B) that constitute binding sites for SH3 domain-containing proteins and contain the consensus PXXP (SEQ ID NO:16) sequence that is present in all high affinity SH3 ligands identified to date (Cohen et al, Cell 80:237 (1995); Rickles et al, EMBO J. 13:5598 (1994)). A polyproline stretch is present **upstream** of the SH3 domain which could function as a transcriptional **activation** domain (Tanaka et al, Mol. Cell. Biol. 14:6046 (1994)). Sequences enriched in serine/threonine, glutamate/aspartate and proline residues, designated PEST regions, are also found in the present protein. PEST regions are identified in the central and C-terminal portions of the Abi protein (see FIG. 1, B). The N-terminal region of the present protein is basic (eg, calculated pI about 11.4) and homologous to the DNA-binding sequence of homeodomain proteins (the protein of the invention is unique among the family to which it relates in having both an SH3 domain and a homeodomain homologous region). The C-terminal portion of the protein is acidic (eg, pI about 3.5). A serine-rich region is present in the central portion of the protein (see FIG. 1, B). The Abi protein contains several (eg 9) serine/threonine residues followed by proline, indicative of phosphorylation by proline-directed protein kinases (Kemp and Pearson, Trends Biochem. Sci. 15:342 (1990)). Certain sites conform to the cdc 2 kinase consensus sequence Ser/Thr-Pro-X-basic (Moreno and Nurse, Cell 61:549 (1990)). There are also potential cAMP-dependent protein kinase sites (eg 11) and

potential protein kinase C sites (eg 9) (Kemp and Pearson, Trends Biochem. Sci. 15:342 (1990)). Several tyrosines in the sequence are found in peptides that correspond to optimal peptide substrates for the Ab1, Fps and **Src** protein tyrosine kinases (Songyang et al, Nature 373:536 (1995)).

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	4018	src	USPAT; US-PGPUB	2002/03/12 10:58
2	L2	529990	activat$8	USPAT; US-PGPUB	2002/03/12 10:59
3	L3	161431	upstream	USPAT; US-PGPUB	2002/03/12 10:59
4	L4	1746	2 near5 3	USPAT; US-PGPUB	2002/03/12 10:59
5	L5	8	4 same 1	USPAT; US-PGPUB	2002/03/12 11:00
6	L6	20	1 same 2 same 3	USPAT; US-PGPUB	2002/03/12 11:01
7	L7	99	1 near2 (activator$1 or activation)	USPAT; US-PGPUB	2002/03/12 11:02
8	L8	1856	hbv or hbx	USPAT; US-PGPUB	2002/03/12 11:02
9	L9	2	7 and 8	USPAT; US-PGPUB	2002/03/12 11:03

US-PAT-NO: 6274788
DOCUMENT-IDENTIFIER: US 6274788 B1
TITLE: Bicistronic DNA construct comprising X-myc transgene for use in production of transgenic animal model systems for human hepatocellular carcinoma and transgenic animal model systems so produced
DATE-ISSUED: August 14, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kumar; Vjay	New Delhi	N/A	N/A	INX
Singh; Mahavir	New Delhi	N/A	N/A	INX
Totey; Satish	New Delhi	N/A	N/A	INX
Anand; Rajesh	New Delhi	N/A	N/A	INX

US-CL-CURRENT: 800/18,536/23.5 ,536/24.33 ,800/3 ,800/8
ABSTRACT:
The present invention relates to a bicistronic DNA construct comprising X-myc transgene. In particular, the present invention relates to a bicistronic X15-myc transgene capable of expressing truncated X protein and a full-length murine c-myc protein. More particularly, the present invention relates to a bicistronic DNA construct being an X15-myc transgene for use in the production of transgenic animal model systems for human hepatocellular carcinoma and transgenic animal model systems so produced. The invention is based partially on the discovery that in susceptible transgenic mice that carry a bicistronic X-myc transgene there is an accelerated formation of liver tumors involving all lobes.
5 Claims, 12 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

DATE FILED: February 2, 1999

---------- KWIC --------

BSPR:
Hepatocellular carcinoma (HCC) is one of the ten most common human cancers with over 250,000 new cases worldwide each year. Evidence gathered over decades of epidemiological studies clearly indicate that there is an indisputable association between infection due to hepatitis B virus (**HBV**) and HCC. The incidence of HCC is directly proportional to that of **HBV**. At least 50% of individuals chronically infected by **HBV** develop HCC. At present more than 200 million people worldwide are chronically infected. Every year one to two million die as a result of the infection, approximately 700,000 of such deaths being due to **HBV** associated HCC (Szmuness, 1978, Prog. Med. Virol. 24:40-69).

BSPR:
Although an **HBV** vaccine exists, the WHO estimates that 400 million people will be chronically infected by **HBV** by the year 2000. Since, the incubation period

for the development of **HBV**-associated HCC is as long as 30 years or even more, the danger posed by **HBV** related HCC will continue to remain a major threat for decades. Therefore, there is an urgent need for better therapies to supplement existing ones such as liver resection, transplantation and ethanol injection. Otherwise, the situation is not likely to improve. However, it has been difficult to examine the pathogenic mechanism in great detail because of the limited host range of **HBV** and the lack of in vitro culture systems to propagate it. In view of this, most of the studies of HCC were, until recently, limited to the analysis of **HBV**-infected patients and chimpanzees or **HBV**-related hepadnavirus infections in woodchucks.

BSPR:
The close relationship between **HBV** and HCC has made it one of the most attractive and useful animal models for exploring the role of viruses in cancer development. The **HBV** genome has been elucidated and the viral genes implicated in hepatopathogenesis have been characterized. Insertional mutagenesis leading to the activation/inactivation of growth regulatory genes or oncogenes as well as transactivation by viral gene products have been suggested as possible mechanisms of **HBV** associated carcinogenesis. The integration of **HBV** DNA does not show site preferences in the human genome. Nevertheless, it has been reported to integrate in the vicinity of some important cellular genes, e.g., cyclin A (Wang et al., 1990, Nature 343:555-557), retinoic acid receptors (Dejean et at., 1986, Nature 322:70-72) and oncogene hst-1 (Hatada et al., 1988, Oncogene 3:537-540). However, in case of woodchuck hepatitis virus, insertional activation of a myc gene has been observed in more than 70% of the liver tumors (Quignon et at., Oncogene 12:2011-2017).

BSPR:
The sequence coding for the X protein appears to play a very important role in the physiological events leading to cell transformation. A majority of patients who are seronegative for HBsAg, on evaluation by RT-PCR for transcripts of HBsAg, HBcAg and **HBx**, show positivity only for **HBx** transcripts, clearly indicating that the master molecules of **HBV**-mediated transformation is **HBx** (Paterlini et al., Hepatology 21:313-321). It also suggests that the integrated X gene may be important for maintaining the tumor phenotype. Further, **HBx** has been shown to transactivate a variety of viral and cellular promoters (Caselmann, 1996, Adv. Virus Res. 47:253-302) and modulate the tumor promoting pathways (Kekule et al., 1993, Nature 361:742-745; Chirillo et al., 1996, J. Virol. 70:641-646; Klein et al., 1997, Mol. Cell. Biol. 17:6427-6436). **HBx** binds the tumor suppressor p53 protein and disrupts the process of apoptosis (Wang et al., 1995, Cancer Res. 55:6012-6012). This action of **HBx** is found to interfere with the normal surveillance mechanism for removing abnormal cells. Cells with a survival advantage could be selected that in turn may trigger the multi-step process of hepatocarcinogenesis. **HBx** expression can transform NIH3T3 cells as well as a rodent hepatocyte cell line, FMH202 (Schaefer and Gerlich, 1995, intervirology 38:153-154). However, the cell-based transformation studies using **HBx** have run into trouble because these cells are quite often reported to lose their immortalized status (S. Schaefer, Personal communication). Thus, it has been extremely difficult to examine the pathogenetic mechanisms of **HBV** from cell culture studies and there is an urgent need for developing a proper and effective animal model system for such studies.

BSPR:
With the advent of embryo microinjection technology, it became evident that many questions related to **HBx**-associated pathogenesis might be directly examined by introduction of the X gene into transgenic mice. First, the **HBx** transgenic mouse line was generated in the outbred CD1 background in which the X gene was introduced under its natural promoter. High level expression of **HBx** induced progressive changes in the liver beginning with neoplastic lesions, through benign adenomas, and finally to malignant carcinomas that killed most male animals before 15 months of age (Kim et al., 1991, Nature 351:317-320; Koike et al., 1994, Hepatology 19:810-819). Though, these studies demonstrate the oncogenic potential of **HBx,** others have not observed the induction of HCC in independently developed X gene transgenic mouse strains. (Lee et al., 1990, J. Virol. 64:5939-5947; Perfumo et al., 1992, J. Virol. 66:6819-6823). This discrepancy might be associated with the promoter strength, duration of **HBx** expression and genetic backgrounds on which the various transgenic models were produced. This is substantiated by the fact that the mice that develop HCC were produced and maintained on CD-1 background which shows a high spontaneous rate of HCC (Homburger et al., 1975, J. Natl. Cancer. Inst. 55:37-45). This might also suggest that **HBx** might not be sufficient to induce HCC by itself but rather, it functions as a cofactor in the process of hepatopathogenesis. It is therefore, clear that other genetic and epigenetic events and factors are necessary for HCC to develop. In this respect, a significant acceleration of the tumorigenic process was seen in a genetic cross between the **HBx** transgenic and the WHV/c-myc transgenic mice (Terradillos et al., 1997, 14:395-404), but still not as fast as the pathogenetic studies demanded.

BSPR:
The present invention relates to a novel bicistronic DNA construct represented as X-myc transgene useful for raising animal models for HCC. In a preferred embodiment, the DNA construct is X15-myc transgene having the potential to express a truncated X protein (X15, having from 58 to 154 amino acids) that encompasses the minimal transactivation domain of **HBx** (Kumar et al., 1996, Proc. Natl. Acad. Sci. USA 93:5647-5652). In addition, it can express a major form of the full-length murine c-myc protein. The reasons for choosing myc gene were (a) selective amplification of c-myc gene in the **HBV** related HCC cases (Peng et al., 1993, J. Formos. Med. Assoc. 92:866-870) and (b) frequent activation of both c-myc gene and N-myc gene after integration of the viral DNA (Moroy et al., 1986, Nature 324:276-279; Fourel et al., 1990, Nature 347:294-298). Preferably, the X15 region is positioned 5' to the murine c-myc gene and is operatively linked to and under the regulatory control of its natural promoter and enhancer I element. The c-myc gene is operatively linked to and driven by the core promoter and enhancer II elements. The construct of the present invention is rather compact, especially in view of the fact that core promoter and enhancer II regions are embodied in the X gene sequence. No prior art known to the applicants discloses the bicistronic DNA construct i.e., X-myc transgene of the present invention. The transgenic animals of the present invention carrying such transgene develop tumors of the liver within 12-20 weeks of age, considerably faster than any transgenic animal model available. At an extremely early age itself, the transgenic animals show progressive changes in the liver as revealed by histological examinations, beginning with neoplastic lesions to benign adenomas and finally full blown

malignant carcinoma within 12 to 20 weeks of age. Animals of either sex are affected and large tumors develop in all lobes of the liver. Animal models developed earlier in the out bred CD1 background exhibit a much delayed HCC resulting in the death of male mice between 11 to 15 months of age and female mice between 17 to 21 months of age (Kim et al., 1991, 351:317-320). Thus, the transgenic animal model systems for HCC of the present invention are superior to any transgenic animal model system for HCC known in the art in that the transgenic animals of the present invention develop more aggressive and accelerated onset of malignant liver tumors in all lobes causing death of the affected animals in 20 to 22 weeks, i.e., faster than the time it takes the other known transgenic animals to even develop a tumor.

BSPR:
There could be several reasons for the delay in the onset of liver cancer in the Terradillos transgenic mice. Without wishing to be bound by theory, the applicants believe that it is the lack of proximity of the X gene or the flanking regulatory sequences to the c-myc gene that causes delay in the onset of liver cancer. In spite of recognizing this fact, it has hitherto not been possible to produce a bitransgenic mice having the X gene or the flanking regulatory sequence fused with or even sufficiently close to the c-myc gene for the former to influence the latter sufficiently early to bring about an accelerated onset of liver cancer. Neither has it been possible to have the two transgenes integrated on the same chromosome, close enough to each other, to allow manifestation of accelerated onset of liver cancer. In fact, it is practically impossible for a genetic recombination to achieve such proximity, leave alone an integrated construct for the reason that in the WHV/c-myc transgene the X open reading frame and the core promoter/enhancer II elements are missing. In addition, the C-myc gene has three exons, all of which are driven by its own P1 promoter. Secondly, in the **HBx** transgenic mice the X gene has been placed under three independent promoters thereby, further reducing the probability of recombination. Thirdly, the respective transgenes are invariably located on different chromosomes in the two parental or founder transgenic mice. Consequently, the two transgenes are never integrated in the same chromosome in the descendants, as a result of which these transgenic mice always develop a highly delayed onset of hepatocellular carcinoma. Even hypothetically assuming that two founder mice carry their respective transgenes on identical chromosomes, the descendants will still have the trangenes located on different loci, even on the same chromosome. Such lack proximity again causes delay in the onset of liver cancer. As far as the applicants are aware, there is no evidence that the two transgenes have ever been integrated in the same chromosome of any transgenic mice. It was realized by the applicants, for the first time, that the proximity of X gene to c-myc gene played a crucial role in the accelerated onset of hepatocellular carcinoma and that the only way the closest proximity could be achieved was by bringing together the X gene, preferably, the truncated X15 version and the murine c-myc gene outside the mice as a bicistronic X15-c myc construct transgene and thereafter, introducing such transgene into the mice or ancestors thereof at an embryonic stage.

BSPV:
(a) **HBx** transgenic mice (two lines: PEX7 and AX-16): These mice carry a transgene having two or three tandem repeat of the **HBx** gene under the control of either X promoter, core promoter or a erithrombin III core promoter. These

animals do not develop any pathology over two years of observation.

DRPR:
FIG. 1 discloses a diagrammatic representation of the regulatory elements in the **HBV** genome (adw sub type).

DEPR:
The regulatory elements, in the **HBV** genome are diagrammatically illustrated in FIG. 1. Restriction sites are shown in the Figure in which D is DraI site, Bg is Bgl II site. Xp is the natural X promoter which controls the X protein in the construct (FIG. 2) of the invention while Cp is the core promoter which drives the myc gene of the construct. DR1 and DR2 represent the direct repeats 1 and 2 respectively in the X open reading frame.

DEPV:
(b) a 539 bp DraI-Nco I fragment of the **HBV** genome (adw sub type) encompassing the enhancer I and X promoter regions was joined by conventional methods with the c-myc (2+3) gene to generate Xp-myc gene.

DEPV:
(c) a 362 bp fragment of the **HBV** genome encompassing the coding region for the C-terminal half of the X gene having from 58 to 154 amino acids along with enhancer II and core promoter regions was PCR amplified. This was then cloned as Nco I fragment in the Xp-myc construct to generate X15-myc construct. The following two primers were employed in the polymeric chain (PCR) reaction:

CLPR:
1. A bicistronic hepatitis B virus (**HBV**) X15-c-myc transgene, wherein said **HBV** X15-c-myc transgene comprises in sequence: a **HBV** X15 transgene and a c-myc transgene;

CLPR:
2. A transgenic mouse wherein the germ cells and somatic cells of the transgenic mouse comprise a bicistronic **HBV** X15-c-myc transgene, wherein said **HBV** X15-c-myc transgene comprises in sequence: a **HBV** X15 transgene and a c-myc transgene;

CLPR:
4. A method of making a bicistronic **HBV** X15-c-myc transgene DNA construct comprising

CLPV:
wherein said bicistronic **HBV** X15-c-myc transgene encodes a truncated **HBV** X15 protein comprising amino acids 58-154 of **HBV** X15 and a murine c-myc protein, wherein said **HBV** X15 transgene comprises the nucleotide sequences disclosed in SEQ ID NO:1 and said murine c-myc transgene comprises exons 2 and 3 of murine c-myc;

CLPV:
wherein said **HBV** X15 transgene is operatively linked to and under the regulatory control of its natural **HBV** X15 promoter and enhancer I elements and the c-myc transgene is operatively linked to and under the regulatory control

of a core promoter and an enhancer II element of the HBV X15 gene.

CLPV:
wherein said bicistronic HBV X15-c-myc transgene encodes a truncated HBV X15 protein comprising amino acids 58-154 of HBV X15 and a murine c-myc protein, wherein said HBV X15 transgene comprises the nucleotide sequences disclosed in SEQ ID NO:1 and said murine c-myc transgene comprises exons 2 and 3 of murine c-myc;

CLPV:
wherein said HBV X15 transgene is operatively linked to and under the regulatory control of its natural HBV X15 promoter and enhancer I elements and the c-myc transgene is operatively linked to and under the regulatory control of a core promoter and an enhancer II element of the HBV X15 gene; and

CLPV:
wherein the expression of said HBV X15-c-myc transgene results in development of hepatocellular carcinoma in the transgenic mouse.

CLPV:
(i) providing a transgenic mouse wherein the germ cells and somatic cells of the transgenic mouse comprise a bicistronic HBV X15-c-myc transgene, wherein said HBV X15-c-myc transgene comprises in sequence: a HBV X15 transgene and a c-myc transgene;

CLPV:
(b) joining a 539 base pair Dra I-Nco I fragment of the HBV genome encompassing the enhancer I and X promoter regions said c-myc (2+3) gene to generate Xp-c-myc gene; and

CLPV:
(c) amplifying by polymerase chain reaction (PCR) a 362 base pair fragment of the HBV genome encompassing a coding region for the C-terminal half of the X gene, wherein the coding region encodes amino acids 58-154 of HBVX15, an enhancer II and a core promoter region, and cloning the 263 base pair fragment into the Nco I site of the Xp-c-myc construct to generate the HBV X15-c-myc transgene.

CLPW:
wherein said bicistronic HBV X15-c-myc transgene encodes a truncated HBV X15 protein comprising amino acids 58-154 of HBV X15 and a murine c-myc protein, wherein said HBV X15 transgene comprises the nucleotide sequences disclosed in SEQ ID NO:1 and said murine c-myc transgene comprises exons 2 and 3 of murine c-myc;

CLPW:
wherein said HBV X15 transgene is operatively linked to and under the regulatory control of its natural HBV X15 promoter and enhancer I elements and the c-myc transgene is operatively linked to and under the regulatory control of a core promoter and an enhancer II element of the HBV X15 gene; and

CLPW:

wherein the expression of said **HBV** X15 transgene results in the development of hepatocellular carcinoma in the transgenic mouse;

ORPL:
Kim, C., et al. "**HBx** gene of hepatitis B virus induces liver cancer in transgenic mice." Nature, vol. 351 (May 23, 1991) pp. 317-320.

ORPL:
Koike, K., et al. "High-level Expression of Hepatitis B Virus **HBx** Gene and Hepatocarcinogenesis in Transgenic Mice." Hepatology, vol. 19, No. 4 (1994) pp. 810-819.

ORPL:
Klein, N., et al. "**Activation of Src** Family Kinases by Hepatitis B Virus **HBx** Protein and Coupled Signaling to Ras." Molecular and Cellular Biology, vol. 17, No. 11 (Nov. 1997) pp. 6427-6436.

ORPL:
Kekule, A., et al. "Hepatitis B virus transactivator **HBx** uses a tumour promoter signalling pathway" Nature, vol. 361 (Feb. 25, 1993) pp. 742-745.

US-PAT-NO: 5885833
DOCUMENT-IDENTIFIER: US 5885833 A
TITLE: Nucleic acid constructs for the cell cycle-regulated expression of genes and therapeutic methods utilizing such constructs
DATE-ISSUED: March 23, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mueller; Rolf	Marburg	N/A	N/A	DEX
Zwicker; Joerk	Marburg	N/A	N/A	DEX
Sedlacek; Hans-Harald	Marburg	N/A	N/A	DEX

US-CL-CURRENT: 435/372,435/320.1 ,536/24.1
ABSTRACT:
Nucleic acid constructs comprising an activator sequence, a promoter module, and a structural gene are disclosed. The promoter module comprises a a CHR region and a nucleic acid sequence that binds a protein of the E2F family. These constructs are used in gene therapy, such as the treatment of disorders characterized by excess cell proliferation.
19 Claims, 7 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 7

DATE FILED: February 13, 1997

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BSPR:
In a further embodiment, the invention relates to a nucleic acid construct as described above, wherein said activator sequence (a) is a promoter or enhancer sequence from a virus, wherein said virus is selected from the group consisting of the viruses **HBV,** HCV, HSV, HPV, EBV, HTLV, CMV, SV40 or HIV.

DEPR:
In one embodiment, the activator sequence (a) in the nucleic acid constructs according to the invention is cell-specific or virus-specific or metabolic specific. As used in this specification, "cell-specific" means that the activator sequence is selected from a gene coding for a protein that is specifically expressed in a given cell, and "virus-specific" means that the activator sequence is selected from a viral gene; metabolic specific means, that the activator sequence is selected from a gene, coding for a protein, that is specifically expressed under defined metabolic conditions. Thus in another embodiment, the nucleic acid constructs according to the invention have an activating sequence (a) which is selected from the group of promoters or enhancers which activate transcription in endothelial cells, smooth muscle cells, hemopoietic cells, lymphocytes, macrophages, tumor cells, leukemia cells or glial cells, or from promoter sequences or enhancer sequences of the viruses **HBV,** HCV, HSV, HPV, EBV, HTLV or HIV. Examples of cell-specific activator sequences, virus-specific sequences and metabolic specific sequences are

described below.

DEPR:
The activator sequence to be chosen comprises promoter sequences from cellular genes whose activity is altered in particular by infections with bacteria or parasites, or the promoter sequences to be chosen are those from viruses which transform the cells infected by them and stimulate proliferation. These viruses include, for example, **HBV,** HCV, HSV, HPV, HIV, EBV and HTLV.

CLPR:
16. A nucleic acid construct as claimed in claim 1, wherein said activator sequence (a) is a promoter or enhancer sequence from a virus, wherein said virus is selected from the group consisting of the viruses **HBV,** HCV, HSV, HPV, EBV, HTLV, CMV, SV40 and HIV.

ORPL:
Mukhopadnyay et al., "Hypoxic induction of human vascular endothelial growth factor expression through c-**Src activation,**" Nature 375, (1995), pp. 577-581.

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	4018	src	USPAT; US-PGPUB	2002/03/12 10:58
2	L2	529990	activat$8	USPAT; US-PGPUB	2002/03/12 10:59
3	L3	161431	upstream	USPAT; US-PGPUB	2002/03/12 10:59
4	L4	1746	2 near5 3	USPAT; US-PGPUB	2002/03/12 11:18
5	L5	8	4 same 1	USPAT; US-PGPUB	2002/03/12 11:00
6	L6	20	1 same 2 same 3	USPAT; US-PGPUB	2002/03/12 11:01
7	L7	99	1 near2 (activator$1 or activation)	USPAT; US-PGPUB	2002/03/12 11:02
8	L8	1856	hbv or hbx	USPAT; US-PGPUB	2002/03/12 11:02
9	L9	2	7 and 8	USPAT; US-PGPUB	2002/03/12 11:03
10	L10	273	1 near5 2	USPAT; US-PGPUB	2002/03/12 11:19
11	L11	9	8 and 10	USPAT; US-PGPUB	2002/03/12 11:19

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TITLE: Eukaryotic layered vector initiation systems for production of recombinant proteins
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INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/69.1,435/455 ,536/23.2 ,536/23.72 ,536/24.1
ABSTRACT:
The present invention provides compositions and methods for utilizing recombinant alphavirus vectors. Also disclosed are compositions and methods for making and utilizing eukaryotic layered vector initiation systems.
14 Claims, 37 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 30

DATE FILED: July 8, 1999

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BSPR:
In still other embodiments, the vectors described above include a selected heterologous sequence which may be obtained from a virus selected from the group consisting of influenza virus, HPV, **HBV,** HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and CMV. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5,E6, E7 and L1. In yet other embodiments, the vectors described above include a selected heterologous sequence encoding an HIV protein selected from the group consisting of HIV gp120 and gag.

BSPR:
The selected heterologous sequences described above also may be an antisense sequence, noncoding sense sequence, or ribozyme sequence. In preferred embodiments, the antisense or noncoding sense sequence is selected from the group consisting of sequences which are complementary to influenza virus, HPV, **HBV,** HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and CMV sequences.

BSPR:
Within another aspect, **HBV**-derived and coronavirus-derived packaging cell lines are provided which are suitable for packaging and production of and alphavirus vector. Within one embodiment, an **HBV**-derived packaging cell line is provided, comprising an expression cassette which directs the expression of **HBV** core,

PreS/S, and P proteins. Within another embodiment, a coronavirus-derived packaging cell line is provided, comprising an expression cassette which directs the expression of coronavirus N, M, and S proteins.

DEPR:
Within related aspects of the present invention, alphavirus cell-specific expression vectors may be constructed to express viral antigens, ribozyme, antisense sequences or immunostimulatory factors such as gamma-interferon (.gamma.-IFN), IL-2 or IL-5 for the targeted treatment of virus infected cell types. In particular, in order to target alphavirus vectors to specific foreign organism or pathogen-infected cells, inverted repeats of the alphavirus vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, CMV, **HBV,** HPV and HSV.

DEPR:
Another example of an immunomodulatory cofactor is the B7/BB1 costimulatory factor. Briefly, activation of the full functional activity of T cells requires two signals. One signal is provided by interaction of the antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen-presenting cells. Briefly, the second signal is required for interleukin-2 (IL-2) production by T cells and appears to involve interaction of the B7/BB1 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., J. Exp. Med., 173:721-730, 1991a, and J. Exp. Med., 174:561-570, 1991). Within one embodiment of the invention, B7/BB1 may be introduced into tumor cells in order to cause costimulation of CD8.sup.+ T cells, such that the CD8.sup.+ T cells produce enough IL-2 to expand and become fully activated. These CD8.sup.+ T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7/BB1 factor and, for example, an immunogenic **HBV** core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will becomes more effective antigen-presenting cells. The **HBV** core-specific CTL response will be augmented from the fully activated CD8.sup.+ T cell via the costimulatory ligand B7/BB1.

DEPR:
Within other aspects of the present invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of antigens from foreign organisms or other pathogens. Representative examples of such antigens include bacterial antigens (e.g., E. coli, streptococcal, staphylococcal, mycobacterial, etc.), fungal antigens, parasitic antigens, and viral antigens (e.g., influenza virus, Human Immmunodeficiency Virus ("HIV"), Hepatitis A, B and C Virus ("HAV", "**HBV**" and "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, TTLV I, HTLV II and Cytomegalovirus ("CMV"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (i.e., cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated

through Major Histocompatability Complex ("MHC") class I presentation, MHC Class II presentation, or both.

DEPR:
Within one aspect of the invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of Hepatitis B antigens. Briefly, the Hepatitis B genome is comprised of circular DNA of about 3.2 kilobases in length and has been well characterized (Tiollais et al., Science 213:406-411, 1981; Tiollais et al., Nature 317:489-495, 1985; and Ganem and Varmus, Ann. Rev. Biochem. 56:651-693, 1987; see also EP 0 278,940, EP 0 241,021, WO 88/10301, and U.S. Pat. Nos. 5,696,898 and 5,024,938, which are hereby incorporated by reference). The Hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAgs), an HBc antigen (HBcAg), and HBe antigen (HBeAg), and an **HBx** antigen (HBxAg) (see Blum et al., TIG 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of a P22 pre-core intermediate and is secreted from the cell. HBeAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

DEPR:
As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described S antigens may be combined in order to induce an immune response when administered by one of the alphavirus vector constructs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S open reading from of **HBV,** particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant "a". Mutually exclusive subtype determinants, however, have also been identified by two-dimensional double immunodiffusion (Ouchterlony, Progr. Allergy 5:1, 1958). These determinants have been designated "d" or "y" and "w" or "r" (LeBouvier, J. Infect. 123:671, 1971; Bancroft et al., J. Immunol. 109:842, 1972; and Courouce et al., Bibl. Haematol. 42:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the hepatitis B virus S open reading frame, resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the Hepatitis B virus S open reading frame causes a subtype shift from d to y, and (2) exchange of arginine-160 to lysine causes the shift from subtype r to w. In Africans, subtype ayw is predominant, whereas in the U.S. and northern Europe the subtype adw.sub.2 is more abundant (Molecular Biology of the Hepatitis B Virus, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S open reading frame of **HBV** virus isolated from individuals within that region.

DEPR:
Also presented by **HBV** are pol ("**HBV** pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of **HBV** encodes reverse transcriptase activity

found in virions and core-like particles in infected livers. The polymerase protein consists of at least two domains: the amino terminal domain which encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of **HBV** pol may be determined utilizing methods described herein (e.g., below and in Example 13), utilizing alphavirus vector constructs described below, and administered in order to generate an immune response within a warm-blooded animal. Similarly, other **HBV** antigens, such as ORF 5 and ORF 6 (Miller et al., Hepatology 9:322-327, 1989) may be expressed utilizing alphavirus vector constructs as described herein. Representative examples of alphavirus vector constructs utilizing ORF 5 and ORF 6 are set forth below in the examples.

DEPR:
A wide variety of vector systems may be utilized as the first layer of the eukaryotic layered vector initiation system, including for example, viral vector constructs developed from DNA viruses such as those classified in the Poxviridae, including for example canary pox virus or vaccinia virus (e.g., Fisher-Hoch et al., PNAS 86:317-321, 1989; Flexner et al., Ann. N.Y. Acad. Sci. 569:86-103, 1989; Flexner et al., Vaccine 8:17-21, 1990; U.S. Pat. Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); Papoviridae such as BKV, JCV or SV40 (e.g., Mulligan et al., Nature 277:108-114, 1979); Adenoviridae, such as adenovirus (e.g., Berkner, Biotechniques 6:616-627, 1988; Rosenfeld et al., Science 252:431-434, 1991); Parvoviridae, such as adeno-associated virus (e.g., Samulski et al., J. Vir. 63:3822-3828, 1989; Mendelson et al., Virol 166:154-165, 1988; PA 7/222,684); Herpesviridae, such as Herpes Simplex Virus (e.g., Kit, Adv. Exp. Med. Biol. 215:219-236, 1989); and Hepadnaviridae (e.g., **HBV**), as well as certain RNA viruses which replicate through a DNA intermediate, such as the Retroviridae (see, e.g., U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805; Retroviridae include leukemia in viruses such as MoMLV and immunodeficiency viruses such as HIV, e.g., Poznansky, J. Virol. 65:532-536, 1991).

DEPR:
Within other aspects of the present invention, compositions and methods are provided for administering an alphavirus vector construct which is capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, **HBV** HTLV I, HTLV II, CMV, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

DEPR:
Using techniques described above, the lacZ gene encoding the .beta.-galactosidase reporter protein was cut from the plasmid pSV-.beta.-galactosidase (PROMEGA CORP, Madison, Wis.) and substituted into the ELVIS-luc plasmid DNA vector in place of luciferase. To examine in vivo gene expression from ELVIS vectors, Balb/c mice and rats are injected intramuscularly (i.m.) with ELVIS-.beta.-gal or ELVIS-luc plasmid DNA vectors. FIG. 24 demonstrates the in vivo expression of .beta.-galactocidase in muscle tissue taken from a rat and stained with X-gal at three days post i.m. injection. Mice injected with ELVIS-.beta.-gal also demonstrate positively

staining blue muscle fibers. Luciferase expression levels from muscle which were between 75- and 300-fold higher than control levels were detected in 3/4 Balb/c mice at two days post i.m. inoculation with ELVIS-luc plasmid. In other experiments, C3H/HeN mice were injected i.m. with ELVIS vectors expressing either the hepatitis B virus core (HBV-core) or hepatitis B virus e (HBV-e) proteins. Using ELISA detection systems, both HBV-core- and HBV-e-specific IgG antibodies were detected in serum samples collected from the mice 10 days following the second injection with the vectors. These experiments demonstrate that Sindbis-derived DNA vectors are able to express foreign genes in vivo, in rare and mouse muscle.

DEPR:
In the deviation of alphavirus vector packaging and producer cell lines, many approaches are outlined to control the expression of viral genes, such that producer cell lines stably transformed with both vector and vector packaging cassettes, can be derived. These approaches include inducible and/or cellular differentiation sensitive promoters, antisense structural genes, heterologous control systems, and mosquito or other cells in which viral persistent infections are established. Regardless of the final configuration for the alphavirus vector producer cell line, the ability to establish persistent infection, or at least delay cell death as a result of viral gene expression, may be enhanced by inhibiting apoptosis. For example, the DNA tumor viruses, including adenovirus, HPV, SV40, and mouse polyomavirus (Py), transform cells in part, by binding to, and inactivating, the retinoblastoma (Rb) gene product p105 and its closely related gene product, p107, and other gene products involved in the control of the cell cycle including cyclin. A p33.sup.cdk2 and p34.sup.cdc2. All of these viruses, except for Py, encode gene products which bind to and inactivate p53. Uniquely, Py encodes middle T antigen (mT) which binds to and activates the membrane tyrosine kinase, src, and also phosphatidy-linositol-3-kinase, which is required for the full transformation potential of this virus (Talmage et al., Cell 59:55-65, 1989). The binding to and inactivation of the Rb and p53 recessive oncogene products prevents cells transformed by these DNA tumor viruses from entering the apoptotic pathway. It is known that p53 is able to halt the division of cells, in part by inhibiting the expression of proteins associated with cellular proliferation, including c-fos, hsc70, and bcl-2 (Miyashita et al., Cancer Research 54:3131-3135,1994).

DEPR:
In another embodiment, a RNA packaging signal derived from another virus is inserted into the alphavirus vector to allow packaging by the structural proteins of that corresponding virus. For example, the 137 nt. packaging signal from hepatitis B virus, located between nts. 3134 and 88 and spanning the precore/core junction (Junker-Niepmann et al. EMBO J. 9:3389, 1990), is amplified from an HBV template using two oligonucleotide primers. PCR is performed using a standard three temperature cycling protocol, plasmid pHBV1.1 (Junker-Niepmann et al. EMBO J. 9:3389, 1990) as the template, and the following oligonucleotide pair, each of which contain 20 nucleotides complementary to the HBV sequence and flanking ApaI recognition sequences:

DEPR:
In each case, the packaging sequences from HBV, coronavirus, retrovirus, or any other virus, also may be incorporated into alphavirus vectors at locations

other than those outlined above, provided the location is not present in the subgenomic transcript. For example, the next most preferable site of insertion is the carboxy-terminal region of nonstructural protein 3, which has been shown to be highly variable in both length and sequence among all alphaviruses for which sequence information is available. Further, these applications are not limited by the ability to derive the corresponding packaging cell lines, as the necessary structural proteins also may be expressed using any of the alternative approaches described in Example 8.

DEPR:
1. SITE-DIRECTED MUTAGENESIS OF HBV E/CORE SEQUENCE UTILIZING PCR

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence from SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

DEPR:
The second primer sequence corresponds to the anti-sense nucleotide sequence from the SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

DEPR:
The first PCR reaction corrects the deletion in the antisense strand and the second reaction corrects the deletion in the sense strands. PCR reactions one and two correct the mutation from CC to CCA which occurs in codon 79 and a base pair substitution from TCA to TCT in codon 81. Primer 1 contains two consecutive Xho I sites 10 bp upstream of the ATG codon of HBV e coding region and primer 4 contains a Cla I site 135 bp downstream of the stop codon of HBV precore/core coding region. The products of the first and second PCR reactions are extended in a third PCR reaction to generate one complete HBV precore/core coding region with the correct sequence.

DEPR:
2. ISOLATION OF HBV CORE SEQUENCE

DEPR:
The PCR product from the third reaction yields the correct sequence for HBV precore/core coding region.

DEPR:
To isolate HBV core coding region, a primer is designed to introduce the Xho I restriction site upstream of the ATG start codon of the core coding region, and eliminate the 29 amino acid leader sequence of the HBV precore coding region. In a fourth reaction, the HBV core coding region is produced using the PCR product from the third reaction and the following two primers.

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence for the T-3 promoter present in the SK.sup.+ HBe plasmid. The approximately 600 bp PCR product from the fourth PCR reaction contains the HBV core coding region and novel Xho I restriction sites at the 5' end and Cla I restriction sites at the

3' end that was present in the multicloning site of the SK.sup.+ HBe plasmid.

DEPR:
Following the fourth PCR reaction, the solution is transferred into a fresh 1.5 ml microfuge tube. Fifty microliters of 3 M sodium acetate is added to this solution followed by 500 .mu.l of chloroform:isoamyl alcohol (24:1). The mixture is vortexed and then centrifuged at 14,000 rpm for 5 minutes. The aqueous phase is transferred to a fresh microfuge tube and 1.0 ml 100% EtOH is added. This solution is incubated at -20.degree. C. for 4.5 hours, and then centrifuged at 10,000 rpm for 20 minutes. The supernatant is decanted, and the pellet rinsed with 500 .mu.l of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum and then resuspended in 10 .mu.l deionized H.sub.2 O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The approximately 600 bp Xho I-Cla I **HBV** core PCR fragment is cloned into the Xho I-Cla I site of SK.sup.+ plasmid. This plasmid is designated SK+HBc.

DEPR:
3. ISOLATION OF **HBV** X ANTIGEN

DEPR:
4. CONSTRUCTION OF SINDBIS VECTORS EXPRESSING HBVE, **HBV** CORE AND **HBV** X

DEPR:
Construction of a Sindbis vector expressing the **HBV** core sequence is accomplished by digestion of plasmid SK+HBc (described above) with Xho I and Xba I. The HBc fragment is isolated by agarose gel electrophoresis, purified by GENECLEAN.TM. and ligated into pKSSINBV at the Xho I and Xba I sites. This Sindbis-HBc vector is designated pKSSIN-HBc.

DEPR:
Construction of a Sindbis vector expressing the **HBV**-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Xba I to release a cDNA fragment encoding **HBV**-X sequences. The fragment is isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pKSSINBV, pre-treated with Xho I and Xba I enzymes. This Sindbis-**HBx** vector is designated pKSIN-**HBx**.

DEPR:
The above Sindbis **HBV** expressing vectors may also be modified to coexpress a selectable drug resistance marker dependent on the requirements of the experiment or treatment of the vector infected cells. In particular, any of the above Sindbis **HBV** expression vectors described may also be designed to coexpress G418 resistance. This is accomplished by incorporating an internal ribosomal entry site (Example 5) followed by the bacterial noemycin phosphotransferase gene placed 3' of the **HBV** coding sequences and 5' of the terminal 3' end of the vector using the multiple cloning site of the vector. These G418 resistant vector constructs can be used for selecting vector infected cells for the generation of **HBV** specific CTL targets in the following sections.

DEPR:

Cell lysates from cells infected by any of the HBV expressing vectors are made by washing 1.0.times.10.sup.7 cultured cells with PBS, resuspending the cells to a total volume of 600 .mu.l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

DEPR:
As shown in FIG. 16, using these procedures approximately 100-200 ng/ml HBV e antigen is expressed in the cell lysates and 300-400 ng/ml HBV e antigen is secreted from BHK cells infected with the Sin BV HB e vector.

DEPR:
As shown in FIG. 17, using these procedures, approximately 40 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 BHK cells infected with the Sin BV HBcore. Mouse fibroblast cells infected with the recombinant HBcore Sindbis vector express 6-7 fold higher HBV core protein levels than the recombinant HBcore retroviral vector transduced cells (WO 93/15207). As shown in FIG. 18, using these procedures, approximately 12-14 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 L-M(TK-) cells infected with the SinBVHBcore vector as compared to the approximately 2 ng/ml HBV core antigen expressed from recombinant HBcore retroviral vector transducer cells.

DEPR:
Using these procedures, it can be shown that CTLs to HBV e antigen can be induced.

DEPR:
Lymphoblastoid cell lines (LCL) are established for each patient by infecting (transforming) their B-cells with fresh Epstein-Barr virus (EBV) taken from the supernatant of a 3-week-old culture of B95-8, EBV transformed marmoset leukocytes (ATCC CRL 1612). Three weeks after EBV-transformation, the LCL are infected with Sindbis vector expressing HBV core or e antigen and G418 resistance. Vector infection of LCL is accomplished by infecting LCL cells with packaged alphavirus vector particles produced from the appropriaste cell line The culture medium consists of RPMI 1640, 20% heat inactivated fetal bovine serum (Hyclone, Logan, Utah), 5.0 mM sodium pyruvate and 5.0 mM non-essential amino acids. Infected LCL cells are selected by adding 800 .mu.g/ml G418. The Jurkat A2/K.sup.b cells (L. Sherman, Scripps Institute, San Diego, Calif.) are infected essentially as described for the infection of LCL cells.

DEPR:
Humoral immune responses in mice specific for HBV core and e antigens are detected by ELISA. The ELISA protocol utilizes 100 .mu.g/well of recombinant HBV core and recombinant HBV e antigen (Biogen, Geneva, Switzerland) to coat 96-well plates. Sera from mice immunized with vector expressing HBV core or HBV e antigen are then serially diluted in the antigen-coated wells and incubated for 1 to 2 hours at room temperature. After incubation, a mixture of rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 with equivalent titers is added to the wells. Horseradish peroxidase ("HRP")-conjugated goat anti-rabbit anti-serum is added to each well and the samples are incubated for 1 to 2 hours

at room temperature. After incubation, reactivity is visualized by adding the appropriate substrate. Color will develop in wells that contain IgG antibodies specific for HBV core or HBV e antigen.

DEPR:
Antigen induced T-helper activity resulting from two or three injections of Sindbis vector expressing HBV core or e antigen, is measured in vitro. Specifically, splenocytes from immunized mice are restimulated in vitro at a predetermined ratio with cells expressing HBV core or e antigen or with cells not expressing HBV core or e antigen as a negative control. After five days at 37.degree. C. and 5% CO.sub.2 in RPMI 1640 culture medium containing 5% FBS, 1.0 mM sodium pyruvate and 10.sup.-5 2-mercaptoethanol, the supernatant is tested for IL-2 activity. IL-2 is secreted specifically by T-helper cells stimulated by HBV core or e antigen, and its activity is measured using the CTL clone, CTLL-2 (ATCC TIB 214). Briefly, the CTLL-2 clone is dependent on IL-2 for growth and will not proliferate in the absence of IL-2. CTLL-2 cells are added to serial dilutions of supernatant test samples in a 96-well plate and incubated at 37.degree. C. and 5%, CO.sub.2 for 3 days. Subsequently, 0.5.mu. Ci.sup.3 H-thymidine is added to the CTLL-2 cells. 0.5Ci .sup.3 H-thymidine is incorporated only if the CTTL-2 cells proliferate. After an overnight incubation, cells are harvested using a PHD cell harvester (Cambridge Technology Inc., Watertown, Mass.) and counted in a Beckman beta counter. The amount of IL-2 in a sample is determined from a standard curve generated from a recombinant IL-2 standard obtained from Boehringer Mannheim (Indianaopolis, Ind.).

DEPR:
The mouse system may also be used to evaluate the induction of humoral and cell-mediated immune responses with direct administration of Sindbis vector encoding HBV core or e antigen. Briefly, six- to eight-week-old female C3H/He mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (ip) with 1.0 ml of lyophilized HBV core or HBV e expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a.

DEPR:
The data generated in the mouse system described above is used to determine the protocol of administration of vector in chimpanzees chronically infected with hepatitis B virus. Based on the induction of HBV-specific CTLs in mice, the subjects in chimpanzee trials will receive four doses of vector encoding core or e antigen at 7 day intervals given in two successively escalating dosage groups. Control subjects will receive a placebo comprised of formulation media. The dosage will be either 10.sup.7 or 10.sup.8 pfu given in four 1.0 ml injections i.m. on each injection day. Blood samples will be drawn on days 0, 14, 28, 42, 56, 70, and 84 in order to measure serum alanine aminotransferase (ALT) levels, the presence of hepatitis B e antigen, the presence of antibodies directed against the hepatitis B e antigen, serum HBV DNA levels and to assess safety and tolerability of the treatment. The hepatitis B e antigen and antibodies to HB e antigen is detected by Abbott HB e rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.) and the serum HBV DNA levels

is determined by the Chiron bDNA assay. Efficacy of the induction of CTLs against hepatitis B core or e antigen can be determined as in Example 13E 1.c.

DEPR:
Based on the safety and efficacy results from the chimpanzee studies, the dosage and inoculation schedule is determined for administration of the vector to subjects in human trials. These subjects are monitored for serum ALT levels, presence of **HBV** e antigen, the presence of antibodies directed against the **HBV** e antigen and serum **HBV** DNA levels essentially as described above. Induction of human CTLs against hepatitis B core or e antigen is determined as in Example 13E 1.c.

DEPR:
1. CONSTRUCTION OF ELVIS VECTORS EXPRESSING HBVE-C, **HBV** CORE AND **HBV** X

DEPR:
Construction of an ELVIS vector expressing the **HBV** e antigen is accomplished by digesting the SK.sup.+ HB e-c plasmid with Xho I and Not I to release the cDNA fragment encoding HBVe-c sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector , previously prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBe.

DEPR:
The HBcore PCR product described previously is digested with Xho I and Cla I, isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and ligated into SK+II (Bluescript, Stratagene, Calif.) digested with Xho I and Cla I. This construct is designated SK+HBcore. Construction of the ELVIS vector expressing the **HBV** core sequence is accomplished by digesting the SK.sup.+ HBcore plasmid with Xho I and Not I to release the cDNA fragment encoding HBVcore sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBcore.

DEPR:
Construction of the ELVIS vector expressing the **HBV**-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Not I to release the cDNA fragment encoding **HBV**-X sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN, and inserted into the pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-**HBX**.

DEPR:
Any of the above three constructs can be used for selecting vector infected cells for the generation of **HBV** specific CTL targets in the following sections.

DEPR:
The pVGELVIS-HBe plasmid DNA is isolated and purified, and 2 ug of pVGELVIS-HBe DNA is complexed with 10 ul of LIPOFECTAMINE.TM. and transfected into 2.times.10.sup.5 BHK cells contained in 35 mM petri plates. Two days post-transfection, supernatants and whole cell lysates were collected and an

ELISA assay (see below) was used to determine the amount of expressed **HBV**-e antigen.

DEPR:
As shown in FIG. 19, using these procedures, approximately 2 ng/ml **HBV** e antigen is expressed in the cell lysates and also secreted from BHK cells transfected with different clones of the pVGELVISHBe plasmid.

DEPR:
The mouse model system is also used to evaluate the induction of humoral and cell-mediated immune responses following direct administration of ELVIS vector expressing **HBV** core or e antigen. Briefly, six- to eight-week-old female Balb/c, C57B1/6, C3H/He mice (Charles River, Mass.) and HLA A2.1 transgenic mice (V. Engelhard, Charlottesville, Va.) are injected intramuscularly (i.m.) with, for example, 50 ug or greater, pVGELVIS-HBcore, pVGELVIS-HBVe or pVGELVIS-**HBX** vector DNA. Two injections are given one week apart. Seven or fourteen days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a. Detection of humoral immune responses in mice is performed essentially as described in Example 13E 2 and detection of T cell proliferation in mice is performed essentially as described in Example 13E 3.

DEPL:
Following amplification, the PCR amplicon is digested with ApaI and purified from a 1.5% agarose gel using MERMAID.TM. (Bio101). Sindbis vector plasmid pKSSINdlJRsjrc (Example 3) also is digested with ApaI, under limited conditions to cleave at only one of its two sites, followed by treatment with CIAP, purification from a 1% agarose gel, and ligation with the above-synthesized **HBV** amplicon, to produce a construct designated pKSSINhbvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Cell lines which express the **HBV** core, preS/S, and P proteins necessary for packaging of the RNA sequence are derived by modification of helper plasmid pCH3143 (Junker-Niepmann et al., EMBO J. 9:3389, 1990) to include a selectable marker. An expression cassette containing the neomycin resistance marker is obtained by digestion of plasmid pBK-RSV (Stratagene) with Mst II and blunt-ending with Klenow fragment. The selectable marker is then ligated into any of several unique sites within pCH3143 that have been digested and their termini made blunt. The resulting construct is transfected into a desired cell line, for example, mouse hepatoma line Hepa1-6 (ATCC #CRL1830), and selected using the drug G418, as described in Example 7. Introduction of the pKSSINhbvJR vector, or related RNA- or DNA-based alphavirus vectors, results in the production of packaged vector particles with the same hepatotropism as **HBV**.

DEPL:
Following amplification, the PCR amplicon is digested with ApaI, purified from a 1.5% agarose gel using MERMAID.TM., and ligated into pKSSINdlJRsjrc, prepared as described for **HBV**. The resulting construct is designated pKSSINmhvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Packaging of vectors modified with this MHV sequence is accomplished by using expression cassettes which produce each of the required coronavirus structural proteins: nucleocapsid (N protein; Armstrong et al., NAR 11:883, 1983); membrane (M protein, Armstrong et al., Nature 308:751, 1984); and spike

(S protein, Luytjes et al., Virology 161:479, 1987). Preferably, these proteins are inserted into the vector-inducible pVGELVSdl-linker plasmid (described previously in this example) and selected for with the G418 drug following transfection into the appropriate cell type. Other expression methodologies (see Example 7) may also be readily utilized. Additional coronaviruses, for example, human coronaviruses OC43 (ATCC #VR-759) and 229E (ATCC #VR-740), can be readily used in place of MHV to produce packaged recombinant alphavirus particles which show tropism for cells in the respiratory tract.

DEPL:
A. ISOLATION OF **HBV** E/CORE SEQUENCE

DEPL:
G. GENERATION OF ELVIS VECTOR CONSTRUCTS WHICH EXPRESS **HBV** ANTIGENS FOR THE
INDUCTION OF AN IMMUNE RESPONSE

DEPC:
Generation of Vector Constructs Which Express **HBV** Antigens for the Induction of an Immune Response

CLPR:
10. The method according to claim 9 wherein said viral antigen is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, **HBV,** HCV, EBV, HIV, HSV, Fel, V, FIV, Hantavirus, HTLV I, HTLV II, and CMV.

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TITLE: Bicistronic DNA construct comprising X-myc transgene for use in production of transgenic animal model systems for human hepatocellular carcinoma and transgenic animal model systems so produced
DATE-ISSUED: August 14, 2001
INVENTOR-INFORMATION:

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US-CL-CURRENT: 800/18,536/23.5 ,536/24.33 ,800/3 ,800/8
ABSTRACT:
The present invention relates to a bicistronic DNA construct comprising X-myc transgene. In particular, the present invention relates to a bicistronic X15-myc transgene capable of expressing truncated X protein and a full-length murine c-myc protein. More particularly, the present invention relates to a bicistronic DNA construct being an X15-myc transgene for use in the production of transgenic animal model systems for human hepatocellular carcinoma and transgenic animal model systems so produced. The invention is based partially on the discovery that in susceptible transgenic mice that carry a bicistronic X-myc transgene there is an accelerated formation of liver tumors involving all lobes.
5 Claims, 12 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

DATE FILED: February 2, 1999

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BSPR:
Hepatocellular carcinoma (HCC) is one of the ten most common human cancers with over 250,000 new cases worldwide each year. Evidence gathered over decades of epidemiological studies clearly indicate that there is an indisputable association between infection due to hepatitis B virus (**HBV**) and HCC. The incidence of HCC is directly proportional to that of **HBV**. At least 50% of individuals chronically infected by **HBV** develop HCC. At present more than 200 million people worldwide are chronically infected. Every year one to two million die as a result of the infection, approximately 700,000 of such deaths being due to **HBV** associated HCC (Szmuness, 1978, Prog. Med. Virol. 24:40-69).

BSPR:
Although an **HBV** vaccine exists, the WHO estimates that 400 million people will be chronically infected by **HBV** by the year 2000. Since, the incubation period

for the development of HBV-associated HCC is as long as 30 years or even more, the danger posed by HBV related HCC will continue to remain a major threat for decades. Therefore, there is an urgent need for better therapies to supplement existing ones such as liver resection, transplantation and ethanol injection. Otherwise, the situation is not likely to improve. However, it has been difficult to examine the pathogenic mechanism in great detail because of the limited host range of HBV and the lack of in vitro culture systems to propagate it. In view of this, most of the studies of HCC were, until recently, limited to the analysis of HBV-infected patients and chimpanzees or HBV-related hepadnavirus infections in woodchucks.

BSPR:
The close relationship between HBV and HCC has made it one of the most attractive and useful animal models for exploring the role of viruses in cancer development. The HBV genome has been elucidated and the viral genes implicated in hepatopathogenesis have been characterized. Insertional mutagenesis leading to the activation/inactivation of growth regulatory genes or oncogenes as well as transactivation by viral gene products have been suggested as possible mechanisms of HBV associated carcinogenesis. The integration of HBV DNA does not show site preferences in the human genome. Nevertheless, it has been reported to integrate in the vicinity of some important cellular genes, e.g., cyclin A (Wang et al., 1990, Nature 343:555-557), retinoic acid receptors (Dejean et at., 1986, Nature 322:70-72) and oncogene hst-1 (Hatada et al., 1988, Oncogene 3:537-540). However, in case of woodchuck hepatitis virus, insertional activation of a myc gene has been observed in more than 70% of the liver tumors (Quignon et at., Oncogene 12:2011-2017).

BSPR:
The sequence coding for the X protein appears to play a very important role in the physiological events leading to cell transformation. A majority of patients who are seronegative for HBsAg, on evaluation by RT-PCR for transcripts of HBsAg, HBcAg and HBx, show positivity only for HBx transcripts, clearly indicating that the master molecules of HBV-mediated transformation is HBx (Paterlini et al., Hepatology 21:313-321). It also suggests that the integrated X gene may be important for maintaining the tumor phenotype. Further, HBx has been shown to transactivate a variety of viral and cellular promoters (Caselmann, 1996, Adv. Virus Res. 47:253-302) and modulate the tumor promoting pathways (Kekule et al., 1993, Nature 361:742-745; Chirillo et al., 1996, J. Virol. 70:641-646; Klein et al., 1997, Mol. Cell. Biol. 17:6427-6436). HBx binds the tumor suppressor p53 protein and disrupts the process of apoptosis (Wang et al., 1995, Cancer Res. 55:6012-6012). This action of HBx is found to interfere with the normal surveillance mechanism for removing abnormal cells. Cells with a survival advantage could be selected that in turn may trigger the multi-step process of hepatocarcinogenesis. HBx expression can transform NIH3T3 cells as well as a rodent hepatocyte cell line, FMH202 (Schaefer and Gerlich, 1995, intervirology 38:153-154). However, the cell-based transformation studies using HBx have run into trouble because these cells are quite often reported to lose their immortalized status (S. Schaefer, Personal communication). Thus, it has been extremely difficult to examine the pathogenetic mechanisms of HBV from cell culture studies and there is an urgent need for developing a proper and effective animal model system for such studies.

BSPR:
With the advent of embryo microinjection technology, it became evident that many questions related to **HBx**-associated pathogenesis might be directly examined by introduction of the X gene into transgenic mice. First, the **HBx** transgenic mouse line was generated in the outbred CD1 background in which the X gene was introduced under its natural promoter. High level expression of **HBx** induced progressive changes in the liver beginning with neoplastic lesions, through benign adenomas, and finally to malignant carcinomas that killed most male animals before 15 months of age (Kim et al., 1991, Nature 351:317-320; Koike et al., 1994, Hepatology 19:810-819). Though, these studies demonstrate the oncogenic potential of **HBx,** others have not observed the induction of HCC in independently developed X gene transgenic mouse strains. (Lee et al., 1990, J. Virol. 64:5939-5947; Perfumo et al., 1992, J. Virol. 66:6819-6823). This discrepancy might be associated with the promoter strength, duration of **HBx** expression and genetic backgrounds on which the various transgenic models were produced. This is substantiated by the fact that the mice that develop HCC were produced and maintained on CD-1 background which shows a high spontaneous rate of HCC (Homburger et al., 1975, J. Natl. Cancer. Inst. 55:37-45). This might also suggest that **HBx** might not be sufficient to induce HCC by itself but rather, it functions as a cofactor in the process of hepatopathogenesis. It is therefore, clear that other genetic and epigenetic events and factors are necessary for HCC to develop. In this respect, a significant acceleration of the tumorigenic process was seen in a genetic cross between the **HBx** transgenic and the WHV/c-myc transgenic mice (Terradillos et al., 1997, 14:395-404), but still not as fast as the pathogenetic studies demanded.

BSPR:
The present invention relates to a novel bicistronic DNA construct represented as X-myc transgene useful for raising animal models for HCC. In a preferred embodiment, the DNA construct is X15-myc transgene having the potential to express a truncated X protein (X15, having from 58 to 154 amino acids) that encompasses the minimal transactivation domain of **HBx** (Kumar et al., 1996, Proc. Natl. Acad. Sci. USA 93:5647-5652). In addition, it can express a major form of the full-length murine c-myc protein. The reasons for choosing myc gene were (a) selective amplification of c-myc gene in the **HBV** related HCC cases (Peng et al., 1993, J. Formos. Med. Assoc. 92:866-870) and (b) frequent activation of both c-myc gene and N-myc gene after integration of the viral DNA (Moroy et al., 1986, Nature 324:276-279; Fourel et al., 1990, Nature 347:294-298). Preferably, the X15 region is positioned 5' to the murine c-myc gene and is operatively linked to and under the regulatory control of its natural promoter and enhancer I element. The c-myc gene is operatively linked to and driven by the core promoter and enhancer II elements. The construct of the present invention is rather compact, especially in view of the fact that core promoter and enhancer II regions are embodied in the X gene sequence. No prior art known to the applicants discloses the bicistronic DNA construct i.e., X-myc transgene of the present invention. The transgenic animals of the present invention carrying such transgene develop tumors of the liver within 12-20 weeks of age, considerably faster than any transgenic animal model available. At an extremely early age itself, the transgenic animals show progressive changes in the liver as revealed by histological examinations, beginning with neoplastic lesions to benign adenomas and finally full blown

malignant carcinoma within 12 to 20 weeks of age. Animals of either sex are affected and large tumors develop in all lobes of the liver. Animal models developed earlier in the out bred CD1 background exhibit a much delayed HCC resulting in the death of male mice between 11 to 15 months of age and female mice between 17 to 21 months of age (Kim et al., 1991, 351:317-320). Thus, the transgenic animal model systems for HCC of the present invention are superior to any transgenic animal model system for HCC known in the art in that the transgenic animals of the present invention develop more aggressive and accelerated onset of malignant liver tumors in all lobes causing death of the affected animals in 20 to 22 weeks, i.e., faster than the time it takes the other known transgenic animals to even develop a tumor.

BSPR:
There could be several reasons for the delay in the onset of liver cancer in the Terradillos transgenic mice. Without wishing to be bound by theory, the applicants believe that it is the lack of proximity of the X gene or the flanking regulatory sequences to the c-myc gene that causes delay in the onset of liver cancer. In spite of recognizing this fact, it has hitherto not been possible to produce a bitransgenic mice having the X gene or the flanking regulatory sequence fused with or even sufficiently close to the c-myc gene for the former to influence the latter sufficiently early to bring about an accelerated onset of liver cancer. Neither has it been possible to have the two transgenes integrated on the same chromosome, close enough to each other, to allow manifestation of accelerated onset of liver cancer. In fact, it is practically impossible for a genetic recombination to achieve such proximity, leave alone an integrated construct for the reason that in the WHV/c-myc transgene the X open reading frame and the core promoter/enhancer II elements are missing. In addition, the C-myc gene has three exons, all of which are driven by its own P1 promoter. Secondly, in the **HBx** transgenic mice the X gene has been placed under three independent promoters thereby, further reducing the probability of recombination. Thirdly, the respective transgenes are invariably located on different chromosomes in the two parental or founder transgenic mice. Consequently, the two transgenes are never integrated in the same chromosome in the descendants, as a result of which these transgenic mice always develop a highly delayed onset of hepatocellular carcinoma. Even hypothetically assuming that two founder mice carry their respective transgenes on identical chromosomes, the descendants will still have the trangenes located on different loci, even on the same chromosome. Such lack proximity again causes delay in the onset of liver cancer. As far as the applicants are aware, there is no evidence that the two transgenes have ever been integrated in the same chromosome of any transgenic mice. It was realized by the applicants, for the first time, that the proximity of X gene to c-myc gene played a crucial role in the accelerated onset of hepatocellular carcinoma and that the only way the closest proximity could be achieved was by bringing together the X gene, preferably, the truncated X15 version and the murine c-myc gene outside the mice as a bicistronic X15-c myc construct transgene and thereafter, introducing such transgene into the mice or ancestors thereof at an embryonic stage.

BSPV:
(a) **HBx** transgenic mice (two lines: PEX7 and AX-16): These mice carry a transgene having two or three tandem repeat of the **HBx** gene under the control of either X promoter, core promoter or a erithrombin III core promoter. These

animals do not develop any pathology over two years of observation.

DRPR:
FIG. 1 discloses a diagrammatic representation of the regulatory elements in the **HBV** genome (adw sub type).

DEPR:
The regulatory elements, in the **HBV** genome are diagrammatically illustrated in FIG. 1. Restriction sites are shown in the Figure in which D is DraI site, Bg is Bgl II site. Xp is the natural X promoter which controls the X protein in the construct (FIG. 2) of the invention while Cp is the core promoter which drives the myc gene of the construct. DR1 and DR2 represent the direct repeats 1 and 2 respectively in the X open reading frame.

DEPV:
(b) a 539 bp DraI-Nco I fragment of the **HBV** genome (adw sub type) encompassing the enhancer I and X promoter regions was joined by conventional methods with the c-myc (2+3) gene to generate Xp-myc gene.

DEPV:
(c) a 362 bp fragment of the **HBV** genome encompassing the coding region for the C-terminal half of the X gene having from 58 to 154 amino acids along with enhancer II and core promoter regions was PCR amplified. This was then cloned as Nco I fragment in the Xp-myc construct to generate X15-myc construct. The following two primers were employed in the polymeric chain (PCR) reaction:

CLPR:
1. A bicistronic hepatitis B virus (**HBV**) X15-c-myc transgene, wherein said **HBV** X15-c-myc transgene comprises in sequence: a **HBV** X15 transgene and a c-myc transgene;

CLPR:
2. A transgenic mouse wherein the germ cells and somatic cells of the transgenic mouse comprise a bicistronic **HBV** X15-c-myc transgene, wherein said **HBV** X15-c-myc transgene comprises in sequence: a **HBV** X15 transgene and a c-myc transgene;

CLPR:
4. A method of making a bicistronic **HBV** X15-c-myc transgene DNA construct comprising

CLPV:
wherein said bicistronic **HBV** X15-c-myc transgene encodes a truncated **HBV** X15 protein comprising amino acids 58-154 of **HBV** X15 and a murine c-myc protein, wherein said **HBV** X15 transgene comprises the nucleotide sequences disclosed in SEQ ID NO:1 and said murine c-myc transgene comprises exons 2 and 3 of murine c-myc;

CLPV:
wherein said **HBV** X15 transgene is operatively linked to and under the regulatory control of its natural **HBV** X15 promoter and enhancer I elements and the c-myc transgene is operatively linked to and under the regulatory control

of a core promoter and an enhancer II element of the HBV X15 gene.

CLPV:
wherein said bicistronic HBV X15-c-myc transgene encodes a truncated HBV X15 protein comprising amino acids 58-154 of HBV X15 and a murine c-myc protein, wherein said HBV X15 transgene comprises the nucleotide sequences disclosed in SEQ ID NO:1 and said murine c-myc transgene comprises exons 2 and 3 of murine c-myc;

CLPV:
wherein said HBV X15 transgene is operatively linked to and under the regulatory control of its natural HBV X15 promoter and enhancer I elements and the c-myc transgene is operatively linked to and under the regulatory control of a core promoter and an enhancer II element of the HBV X15 gene; and

CLPV:
wherein the expression of said HBV X15-c-myc transgene results in development of hepatocellular carcinoma in the transgenic mouse.

CLPV:
(i) providing a transgenic mouse wherein the germ cells and somatic cells of the transgenic mouse comprise a bicistronic HBV X15-c-myc transgene, wherein said HBV X15-c-myc transgene comprises in sequence: a HBV X15 transgene and a c-myc transgene;

CLPV:
(b) joining a 539 base pair Dra I-Nco I fragment of the HBV genome encompassing the enhancer I and X promoter regions said c-myc (2+3) gene to generate Xp-c-myc gene; and

CLPV:
(c) amplifying by polymerase chain reaction (PCR) a 362 base pair fragment of the HBV genome encompassing a coding region for the C-terminal half of the X gene, wherein the coding region encodes amino acids 58-154 of HBVX15, an enhancer II and a core promoter region, and cloning the 263 base pair fragment into the Nco I site of the Xp-c-myc construct to generate the HBV X15-c-myc transgene.

CLPW:
wherein said bicistronic HBV X15-c-myc transgene encodes a truncated HBV X15 protein comprising amino acids 58-154 of HBV X15 and a murine c-myc protein, wherein said HBV X15 transgene comprises the nucleotide sequences disclosed in SEQ ID NO:1 and said murine c-myc transgene comprises exons 2 and 3 of murine c-myc;

CLPW:
wherein said HBV X15 transgene is operatively linked to and under the regulatory control of its natural HBV X15 promoter and enhancer I elements and the c-myc transgene is operatively linked to and under the regulatory control of a core promoter and an enhancer II element of the HBV X15 gene; and

CLPW:

wherein the expression of said **HBV** X15 transgene results in the development of hepatocellular carcinoma in the transgenic mouse;

ORPL:
Kim, C., et al. "**HBx** gene of hepatitis B virus induces liver cancer in transgenic mice." Nature, vol. 351 (May 23, 1991) pp. 317-320.

ORPL:
Koike, K., et al. "High-level Expression of Hepatitis B Virus **HBx** Gene and Hepatocarcinogenesis in Transgenic Mice." Hepatology, vol. 19, No. 4 (1994) pp. 810-819.

ORPL:
Klein, N., et al. "**Activation of Src** Family Kinases by Hepatitis B Virus **HBx** Protein and Coupled Signaling to Ras." Molecular and Cellular Biology, vol. 17, No. 11 (Nov. 1997) pp. 6427-6436.

ORPL:
Kekule, A., et al. "Hepatitis B virus transactivator **HBx** uses a tumour promoter signalling pathway" Nature, vol. 361 (Feb. 25, 1993) pp. 742-745.